Veterinär-Anatomisches Institut
der Vetsuisse-Fakultät Universität Zürich

Direktor ad interim: Prof. Dr. B. Spiess

Arbeit unter Leitung von Prof. Dr. P. Wild

The Significance of US3 Kinase in Herpes Simplex Virus 1 Envelopment as Revealed by High Resolution Electron Microscopy

Inaugural-Dissertation

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vorgelegt von

Sabine Leisinger
Tierärztin
von Davos Dorf, GR

genehmigt auf Antrag von

Prof. Dr. P. Wild, Referent

Prof. Dr. M. Ackermann, Korreferent

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CONTENT

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1. Abstract

Capsids of herpesviruses acquire tegument and envelope by budding at the inner nuclear membrane. US3, a protein kinase, plays an important role in envelopment together with a complex of viral proteins UL31 and UL34. To clarify the significance of US3 Vero cells were infected with a US3 deletion mutant of herpes simplex virus 1. High resolution electron microscopy revealed dramatic folding of the inner nuclear membrane in combination with deposition of a dense substance at the luminal layer, accumulation of capsids within the nucleus and the nuclear periphery, accumulation of virions containing a dense envelope within the perinuclear space, dilation of nuclear pores and a few capsids within the cytoplasm. These alterations compared to wildtype infected cells indicate retardation of budding and of release of virions out of the perinuclear space, and perturbance of the nuclear envelope. Exposure to Brefeldin A, which disassembles the Golgi complex, led to a reduction of membrane foldings, and a shift of virions from the perinuclear space into adjacent RER cisternae. US3 is hence suggested to be involved in regulation of distribution of membrane constituents needed for budding and intraluminal virus transportation acting possibly at the level of the Golgi complex. Yields of infectious virus was equal to that of wild type virus indicating that capsids acquire the requirements for infectivity, i.e. tegument and glycoproteins, in the course of budding at the inner nuclear membrane.

2. Introduction

2.1. Herpes Simplex Virus Type 1 (HSV-1)

Herpesviruses are composed of four morphological distinct substructures: the inner core containing the viral genomic DNA, the icosahedral capsid built of 162 capsomers, the tegument surrounding the capsid, and the viral envelope with embedded glycoproteins. The way of assembly of these different structures is highly complex. Capsids formed within nuclei are transported to the nuclear periphery. Translocation through the nucleocytoplasmic barrier, envelopment, tegumentation and intracellular transportation are controversially discussed for decades that has led to three main theories.

2.2. The Theories of Viral Egress

2.2.1. Theory of Envelopment, De-Envelopment and Re-Envelopment

This theory assumes that capsids are enveloped by budding at the inner nuclear membrane, de-enveloped by fusion of the primary envelope with the outer nuclear membrane, and finally, re-envelopment at cytoplasmic membranes (Stackpole 1969). The first budding step in herpesvirus maturation at the inner nuclear membrane provides the capsid with a primary envelope (Darlington and Moss 1968; Stackpole 1969). The net result of the budding event is the formation of primary enveloped virions located in the perinuclear space. Capsids of primary virions are translocated into the cytoplasm after fusion of the primary envelope with the outer nuclear membrane (Stackpole 1969). Primary virions as observed in cells infected with the alphaherpesviruses HSV-1, pseudorabies virus (PrV), equine herpes virus 1 (EHV-1) and infectious laryngotracheitis

virus differs in morphology from mature extracellular virions in that the spikes, which are prominent on mature virions, are not detectable by electron microscopy in virions within the perinuclear space (Granzow 2001). The appearance of the primary tegument differs from that in mature virions in that it is closely apposed to the primary envelope in a characteristic electron dense sharply bordered ring structure (Granzow 2001). Primary and secondary virions differ not only in morphology but also biochemically. Differences in the phospholipid composition between mature HSV-1 virions and the nuclear membrane indicated that the latter may not form the final envelope (van Genderen, Brandimarti et al. 1994). The UL31 and UL34 gene products have been shown to be constituents of primary enveloped virions but not of mature virus particles (Klupp, Granzow et al. 2000; Fuchs, Klupp et al. 2002; Reynolds, Wills et al. 2002). Other tegument and envelope proteins present in mature virions have not been detected in primary virus particles (Granzow, Klupp et al. 2004; Mettenleiter 2004). Budding of herpesvirus capsids in the cytoplasm after translocation through the nuclear membrane has been observed early in electron microscopic studies (Siminoff and Menefee 1966; Stackpole 1969; Roffman, Albert et al. 1990). Recently, it has been propagated that this sequential envelopment–de-envelopment–re-envelopment theory (Skepper, Whiteley et al. 2001) constitutes the prototypic herpesvirus maturation pathway (Mettenleiter 2002). Tegumentation may start at two different sites. One site is the translocated capsid into the cytoplasm, the other is the future envelopment site (Mettenleiter 2002), the trans Golgi network.

2.2.2. Theory of Egress by Vesicle Formation

This theory assumes that perinuclear virions exit the perinuclear space by vesicle formation at the outer nuclear membrane (Campadelli-Fiume, Farabegoli

et al. 1991). Virions are then transported within these vesicles to the Golgi apparatus for further maturation (Morgan, Ellison et al. 1954; Poliquin, Levine et al. 1985; Lee, Bao et al. 1987; Torrisi, Di Lazzaro et al. 1992; Gershon, Sherman et al. 1994; Ward, Campadelli-Fiume et al. 1994; Di Lazzaro, Campadelli-Fiume et al. 1995) and then to the plasma membrane where virions are released into the extra cellular space by exocytosis.

2.2.3. Theory of Two Diverse Pathways

Recently, the uneconomic de-re-envelopment theory has been challenged on the basis of the following observations (Wild, Schraner et al. 2002; Leuzinger, Ziegler et al. 2005; Wild, Engels et al. 2005):
i) Virions are located within RER cisternae indicating transportation of virions from the perinuclear space into RER cisternae, ii) the presence of virions within Golgi cisternae, iii) connectivity between RER and Golgi cisternae suggesting intraluminal transportation of virions from the RER into Golgi cisternae, iv) indications for packaging at lateral Golgi cisternae that results in vacuoles containing one or several virions, v) indications for budding at the outer nuclear membrane, membranes of RER, Golgi complex and Golgi derived vacuoles, vi) budding of capsids at narrow Golgi cisternae resulting in small concentric vacuoles containing a single virion, vii) impaired nuclear pores through which capsids may gain direct access to the cytoplasm. On the basis of these facts a novel theory was developed that includes two diverse routes.

Nuclear envelopment:
Capsids bud through the inner nuclear membrane, and are then transported from the perinuclear space via RER cisternae into Golgi cisternae for packaging into transport vacuoles of various size containing one or more virions (Wild,

Schraner et al. 2002; Leuzinger, Ziegler et al. 2005; Wild, Engels et al. 2005). Fusion of the viral envelope with cell membranes of the compartments the virion is transported through is considered likely to be prevented by proteins of an unknown nature that are condensed at the viral envelope. In addition, these substance or substances facilitate transportation. These "antifusion proteins and/or transport proteins" are speculated to be removed from the envelope in Golgi cisternae but remain in the transport vacuoles at low concentrations to prevent fusion of viral envelopes with the vacuolar membranes.

Cytoplasmic envelopment:

Capsids escape the nucleus via impaired nuclear pores (Leuzinger, Ziegler et al. 2005; Wild, Engels et al. 2005), approach Golgi membranes from the cytoplasmic side and induce budding. Since entire Golgi cisternae are involved in budding, a sphere-like structure comprising two membranes arises. The inner membrane becomes the viral envelope, and the outer one becomes the vacuolar membrane. Fusion of the envelope with the vacuolar membrane is considered likely to be prevented by "antifusion" proteins at high concentrations. Alternatively, capsids escaping the nucleus via impaired nuclear pores can bud at the outer nuclear membrane, RER membranes, membranes of dilated Golgi cisternae, and membranes of Golgi derived vacuoles already containing virions. Virions originating by budding at the outer nuclear membrane and RER membranes need to be transported to Golgi cisternae for packaging.

The fact that capsids acquire both tegument (Roffman, Albert et al. 1990; van Drunen Littel-van den Hurk, Garzon et al. 1995) and an envelope containing glycoproteins (Torrisi, Di Lazzaro et al. 1992; Gilbert and Ghosh 1993; Gilbert, Ghosh et al. 1994) do not support the theory that capsids are de-enveloped by fusion of the primary envelope with the outer nuclear membrane for re-envelopment by Golgi membranes.

2.3. Significance of Tegument and Envelope Proteins

Alphaherpesvirus capsids have to collect more than 15 tegument proteins prior to or concomitantly to acquisition of a lipid envelope containing more than 10 glycoproteins (Steven 1997). Betaherpesvirus need to acquire even more different proteins (Gibson 1996). This assembly is a complex process that is only recently starting to unfold. Only a few of the components of the mature tegument and envelope are conserved which makes it difficult to propose a general pathway of virion formation for all herpesviruses.

For envelopment at the nuclear membrane, two virally encoded proteins have been shown to be crucial. These are the products of the UL31 and UL34 genes of HSV-1 (Chang, Van Sant et al. 1997; Roller, Zhou et al. 2000; Reynolds, Ryckman et al. 2001; Reynolds, Wills et al. 2002). Capsids of primary virions may be translocated into the cytoplasm after fusion of the primary envelope with the outer nuclear membrane (Stackpole 1969). The molecular mechanism of this fusion process is unknown. It is also unclear whether the UL34 protein, which so far is the only membrane protein unequivocally identified as a component of primary virions, is capable of mediating this fusion event either alone or in combination with other viral proteins, e.g. UL31 (Roller, Zhou et al. 2000; Reynolds, Ryckman et al. 2001; Fuchs, Klupp et al. 2002; Reynolds, Wills et al. 2002). These two proteins could also function as transport proteins facilitating transportation of virions into RER cisternae (Leuzinger, Ziegler et al. 2005). UL11 (Baines and Roizman 1992), UL20 (Baines, Ward et al. 1991), UL48 (Mossman, Sherburne et al. 2000), and gK (Foster and Kousoulas 1999; Foster, Rybachuk et al. 2003) have been described to be involved in nuclear egress of HSV-1. It has been proposed that the inner shell of the HSV-1 tegument exhibits icosahedral symmetry and part of it anchored specifically at the vertices (five-fold axis) of the capsid (Zhou, Chen et al. 1999). This inner shell was suggested

to contain the UL36 protein, the largest gene product found in the herpesvirus genome (Baer, Bankier et al. 1984; Chee, Bankier et al. 1990). If the UL36 protein forms the innermost layer of tegument, it has to interact with other tegument proteins for virus maturation to proceed. In agreement with their conservation, the UL36 and UL37 play crucial roles in the tegumentation process. The UL31 and UL34 gene products of PrV are present in virus particles in the perinuclear space but are absent from virions at later stages of assembly (Fuchs, Klupp et al. 2002). In contrast, the UL36, UL37, UL46, UL47, UL48 and UL49 tegument proteins are found only in cytoplasmic enveloped virions and mature PrV particles and are suggested to be presumably not recruited during primary envelopment (Fuchs, Klupp et al. 2002; Klupp, Fuchs et al. 2002; Kopp, Klupp et al. 2002; Granzow, Klupp et al. 2004).

Secondary envelopment has been often observed in close proximity to the Golgi apparatus. Whereas it was proposed that the final envelope is derived from early endosomes (Tooze, Hollinshead et al. 1993) or the endoplasmic reticulum-Golgi-intermediate compartment (Sanchez, Sztul et al. 2000) biochemical and morphological evidence indicate that the vesicles, into which budding occurs, are derived from the Golgi apparatus and most likely belong to the trans-Golgi network (Jones and Grose 1988; Severi, Landini et al. 1988; Whealy, Card et al. 1991; Gershon, Sherman et al. 1994; Zhu, Gershon et al. 1995; Whiteley, Bruun et al. 1999; Granzow 2001; McMillan and Johnson 2001). How these vesicles can increase in size to form finally both the viral envelope and the vacuolar membrane remains mysterious (Leuzinger, Ziegler et al. 2005). It is also unclear how tegumented capsids are directed to the envelopment site and how viral glycoproteins are assembled there. However, recently a conserved virion tegument component, the UL11 protein (MacLean, Clark et al. 1989; Harper and Kangro 1990; Sanchez, Sztul et al. 2000; Loomis, Bowzard et al. 2001; Kopp, Granzow et al. 2003; Schimmer and Neubauer 2003), and a conserved viral

glycoprotein, gM (Klupp, Nixdorf et al. 2000), have been implicated in these (Kopp, Granzow et al. 2004) processes. The UL11 might influence secondary envelopment by directing tegument components (Baines and Roizman 1992; Kopp, Granzow et al. 2003; Silva, Yu et al. 2003)and, conceivably, associated capsids to the budding site whereas gM might act by accumulating envelope glycoproteins at these locations.

Envelopment finally results in an enveloped virion within a secretory vesicle. The vesicle is transported to the plasma membrane where the vesicular membrane fuses with plasma membrane releasing the mature virions into the extracellular space. Little is known about the viral components involved in vesicle transportation and exocytosis. UL20 and gK of HSV-1 and PrV have been implicated in this process (Baines, Ward et al. 1991; Avitabile, Ward et al. 1994; Fuchs, Klupp et al. 1997; Foster and Kousoulas 1999).

2.4. Significance of US3

US3, a protein kinase present in HSV-1 infected cells (Purves, Deana et al. 1986; Purves, Katan et al. 1986), is a product of a viral gene. The enzyme is encoded by the open reading frame designated US3 that maps in the unique sequences of the S component of HSV-1 DNA (Purves, Longnecker et al. 1987; Reynolds, Ryckman et al. 2001). UL34 protein is a substrat for the HSV-1 US3-encoded kinase (Purves, Katan et al. 1986; Frame, Purves et al. 1987; Purves, Spector et al. 1991; Purves, Spector et al. 1992; Roller, Zhou et al. 2000; Shiba, Daikoku et al. 2000; Ye and Roizman 2000; Reynolds, Wills et al. 2002). US3 is required for even distribution of the UL31 and UL34 proteins around the nuclear rim of wild-type infected cells. The HSV-2 US3 kinase has been proposed to phosphorylate the UL34 protein, thereby modulating UL34 function (Purves and

Roizman 1992). Mutants lacking the US3 protein kinase was reported to block transportation of capsids from the nucleus to the perinuclear space (Reynolds, Wills et al. 2002) suggesting that the effect of US3 may be on the integrity of the nuclear envelope and not specifically on the mechanism of capsid transportation. In addition, the US3- encoded kinase has been proposed to play a role in protecting HSV-1 infected cells from virus-induced apoptosis (Leopardi, Van Sant et al. 1997; Galvan and Roizman 1998; Asano, Honda et al. 1999; Jerome, Fox et al. 1999; Munger, Chee et al. 2001).

Interestingly, the PrV UL34 protein is phosphorylated to a similar extent in the presence or absence of US3 protein indicating that the observed phenotype of US3 deletion mutants is not due to differences in UL34 phosphorylation (Klupp, Granzow et al. 2000). In the absence of the PrV US3 protein homologue, large numbers of enveloped virions appear to accumulate within invaginations of the nuclear membrane (Wagenaar, Pol et al. 1995; Klupp 2001). The UL34 gene encodes a type II C-terminally anchored membrane protein (Purves, Spector et al. 1992; Klupp, Granzow et al. 2000) that is located in pseudorabies virus PrV infected cells in both leaflets of the nuclear membrane (Klupp, Granzow et al. 2000).

The UL34 gene of HSV-1 (and PRV) encodes a phosphoprotein that is primarily localized at the nuclear envelope of infected cells. It is a necessary component of an envelopment complex that also includes the UL31 protein (Roller, Zhou et al. 2000; Reynolds, Ryckman et al. 2001; Reynolds, Wills et al. 2002). Localization data and sequence analysis suggest that the UL34 protein is anchored within the inner nuclear membrane by a C-terminal hydrophobic domain leaving the bulk of the protein exposed to the interior of the nucleus (Klupp, Granzow et al. 2000; Reynolds, Ryckman et al. 2001; Reynolds, Wills et al. 2002). There are at least three nonmutually exclusive ways by which UL34 protein could facilitate

nuclear egress (Wagenaar, Pol et al. 1995; Klupp 2001; Reynolds, Wills et al. 2002). First, UL34 could directly mediate envelopment through bridging interactions between the capsid and the inner nuclear membrane. Second, the UL34 protein might direct the localization of other important nuclear egress factors. Third, the nuclear lamina, which lines the interior face of the nuclear envelope, may represent a significant physical barrier to nuclear egress. Herpesvirus infection alters this structure (Scott and O'Hare 2001). UL34 may, therefore, affect the architecture of the nuclear lamina to allow capsids to access the envelopment machinery. Data supporting this function have been reported for the UL34 homologue of murine cytomegalovirus (Muranyi, Haas et al. 2002).

The UL31 gene product is a nuclear matrix associated nucleotidylylated phosphoprotein, which in association with the UL34 gene product, localizes to the nuclear rim of HSV-1 infected cells (Chang and Roizman 1993; Blaho, Mitchell et al. 1994; Reynolds, Ryckman et al. 2001; Yamauchi, Shiba et al. 2001; Fuchs, Klupp et al. 2002). The UL31 protein requires UL34 for nuclear membrane targeting (Reynolds, Ryckman et al. 2001; Fuchs, Klupp et al. 2002) whereas the UL34 gene product appears to possess an intrinsic property for targeting to the nuclear membrane (Chang and Roizman 1993). The UL34 protein may thus represent a primary envelope protein whereas the UL31 protein may constitute a primary tegument protein (Mettenleiter 2002).

2.5. Aim of the study

The mutant R7041 was shown to lack the virus induced protein kinase. It was establish that the lack of activity is due the deletion in US3 and not to a fortuitous mutation introduced into the domain of the *tk* gene during the rescue

of the *tk* gene. Concurrently, a mutation in the genes, which partially overlap the *tk* gene, the US3 open reading frame was repaired of recombinant virus R7035 (Purves, Longnecker et al. 1987). R7041 is identical to R7040 except that the 700-base-pair deletion in the *tk* gene was restored by cotransfection of intact R7040 DNA and and *Bam*HI-Q DNA cloned as pRB 103 into rabbit skin cells (Purves, Longnecker et al. 1987).

The goal of this study is to clarify the significance of US3 in HSV-1 envelopment. Therefore, cells were infected with HSV-1 ΔUS3 for analysis by electron microscopy at improved temporal and spatial resolution, which was achieved by rapid freezing of cells after incubation at 37°C for 17 h to 21 h followed by freeze substitution and embedding. This procedure enabled examinations of virus cell interactions in an *in situ* situation with a delay of less than 15 seconds between removal of the sapphire disks from the incubation chamber and freezing (Wild, Schraner et al. 2002). All processes of envelopment at the nuclear envelope, RER, and Golgi complex could be readily visualized due to the well preserved ultrastructure. Close examination of serial sections revealed a dramatic folding of the inner nuclear membrane suggesting de novo synthesis of membrane constituents, and concomitantly, accumulation of budding capsids at the inner nuclear membrane as well as fully enveloped virions within the perinuclear space. Shift of virions from the perinuclear space into RER cisternae and lack of foldings of the inner nuclear membrane after Brefeldin A (BFA) treatment suggest that US3 is involved in membrane synthesis needed for envelopment and, concomitantly, in mediating budding and intraluminal virus transportation.

3. Material and Methodes

3.1. Cells and Viruses

Vero cells (European Collection of Cell Cultures) were grown in Dulbecco's modified minimal essential medium (DMEM; Invitrogen, Basel, Switzerland) supplemented with penicillin (100 U/ml), streptomycin (100 µg/ml) and 10% fetal calf serum (FCS; Omnilab, Mettmenstetten, Switzerland). HSV-1 ΔUS3 was kindly provided by Bernard Roizman, University of Chicago, USA. Wild-type HSV-1 strain F and HSV-1 ΔUS3 were propagated in Vero cells. Virus yields were determined by plaque titration.

3.2. Infection of Cells

Vero cells were grown for 2 days on 30 µm thick sapphire disks with a diameter of 3 mm (Bruegger, Minusio, Switzerland) for low temperature electron microscopy (LTEM), or on cover slips for immunolabeling of nuclear pore proteins. Sapphire disks were covered with 8-10 nm carbon, obtained by evaporation under high vacuum conditions, to enhance cell growth. Cells were infected with HSV-1 ΔUS3 at a multiplicity of infection (MOI) of 5 in 1 ml medium per well in 6 well plates, and kept at 37° C for 1 h to allow adsorption prior to incubation at 37°C for 9 to 21 h.

3.3. Brefeldin A

BFA was added in concentrations of 0.1 µl/ml 5 and 8 h after infection of Vero cells with HSV-1 ΔUS3. The total incubation time after inoculation was 20 h.

3.4. Low-Temperature Transmission Electron Microscopy

Cells grown on sapphire disks were frozen in a high-pressure freezing unit (HPM010; BAL-TEC Inc., Balzers, Liechtenstein) as described in detail (Wild, Schraner et al. 2002). The samples were then transferred to a freeze-substitution unit (FS 7500; Boeckeler Instruments, Tucson, Arizona) precooled to -88°C for substitution with acetone and subsequent fixation with 0.25% glutaraldehyde and 0.5% osmium tetroxide at temperatures between -30°C and +2°C to achieve good contrast of membranes (Wild, Schraner et al. 2001), and embedded in Epon at 4°C followed by polymerization at 60°C for 2.5 days. Serial sections of 60 to 90 nm thickness were analyzed in a transmission electron microscope (CM12; Philips, Eindhoven, The Netherlands) equipped with a slow-scan CCD camera (Gatan, Pleasanton, CA) at an acceleration voltage of 100 kV.

3.5. Morphometric Analysis

Images of Golgi complexes were collected from cells 9, 17 and 21 h after infection with HSV-1 ΔUS3 at MOI 5 or mock infected cells. Volume density and surface density of the Golgi complex were estimated by morphometric analysis on 30 cells selected at random at a final magnification of 89'500 applying the point counting method (Weibel 1979). The data were calculated on the basis of the equations: $V_{vg} = P_g/P_{cy}$, and $S_{vg} = I_g/(P_{cy} \times d)$, whereby V_{vg} is the volume density, S_{vg} the surface density of the Golgi complex, I_g are intersections with Golgi membranes, P_g are points hitting the lumen of the Golgi complex, P_{cy} are points hitting the cytoplasm, and d is the test line length. The data were expressed as μm^3 volume or as μm^2 surface area per 1000 μm^3 cytoplasm.

3.6. Immunolabeling

For demonstration of nuclear pores by confocal microscopy, cells grown on cover slips were infected with HSV-1 ΔUS3 at a MOI 5 and incubated at 37°C. Cells were fixed with 2% formaldehyde in phosphate buffered saline (PBS), pH 7.2, at room temperatur (RT) for 25 min, washed in PBS at 4°C for 5 min, incubated with 50 mM Glycin in PBS at 4°C for 5 min, washed in PBS, treated with 0.1% Triton X-100 (E. Merck, Darmstadt) for 5 to 10 min and, after blocking with 3% bovine serum albumin (BSA) in PBST (PBS and 0.05% Tween 20), incubated with MAb 414 (Convance, Berkeley, California, USA) a mous monoclonal antibody at RT for 1 h. After washing with PBST containing 3% milk, cells were incubated with an anti mouse secondary antibody Alexa Fluor 488 (A 11017; Molecular Probes, Eugene, Oregon, USA). For monitoring virus multiplication cells were then incubated with antibodies against VP16 raised in rabbits (gift from Bernard Roizman, University of Chicago, USA) and finally with a secondary antirabbit antibody conjugated with Alexa Fluor 594 (A 11072, Molecular Probes, Eugene, Oregon, USA). Nuclei were stained with DAPI (Roche). After washing with PBS containing 3% milk and finally with PBS cells were again fixed with 2% formaldehyde for 10 min, washed in water and embedded in Glycergel (DakoCytomation, Carpinteria, USA) with 1,4-Diazabicyclo[2.2.2.] octane (Fluka Chemie GmbH, Buchs, Switzerland). For controls, mock infected cells were used, secondary antibodies were omitted. Samples were analyzed using a confocal laser scanning microscope (SP2, Leica, Mannheim, Germany). Images were deconvolved employing a blind deconvolution algorithm using the program suite Huygens Essential (SVI, Hilversum, The Netherlands).

4. Results

4.1. Nucleus

4.1.1. Nuclear Membranes

Budding of capsids at the inner nuclear membrane is assumed to be inhibited in cells infected with HSV-1 ΔUS3 (Wagenaar, Pol et al. 1995; Klupp, Granzow et al. 2000; Reynolds, Ryckman et al. 2001). To clarify the capsid membrane interactions we investigated the nuclear periphery by electron microscopy at improved temporal and spatial resolution. The most impressive alterations found at the nuclear surface were the numerous capsids budding at the inner nuclear membrane, accumulations of virions within the perinuclear space, and the intense folding of the inner nuclear membrane (Fig. 1-3) forming coil-like structures composed of 1 to 6 layers. These coils developed within the nucleus (Fig. 1 and 2A) or protruded into the cytoplasm (Fig. 1 and 2B). Folds within the nucleus consisted exclusively of the inner membranes (Fig. 2A, 3A and B). The outer membrane seems not to be involved in folding. Folds protruding into the cytoplasm consisted probably also of the inner nuclear membrane. They were delineated to the cytoplasm by the outer nuclear membrane (Fig. 2B). The luminal layer of these folded membranes was always thickened by deposition of an electron dense substance. The space between two luminal layers was often filled by a lesser condensed substance than that on the luminal layer.

Capsids were found to bud at the inner nuclear membrane only at sites that were not thickened (Fig. 2A and 3A). The envelope of all virus particles within the perinuclear space contained a similar dense substance (Fig. 3D, 4A and B) as the inner nuclear membrane. Though many of this particles are detached from the nuclear membrane in a given section plane it cannot be ruled out whether they

are still connected to it above or underneath the section plane. The number of virus particles at budding stages and within the perinuclear space was by a factor 2 lower at 20 and 21 hour compared to 17 h suggesting that virions were possibly transported away from the perinuclear space, and consequently, that budding has been completed.

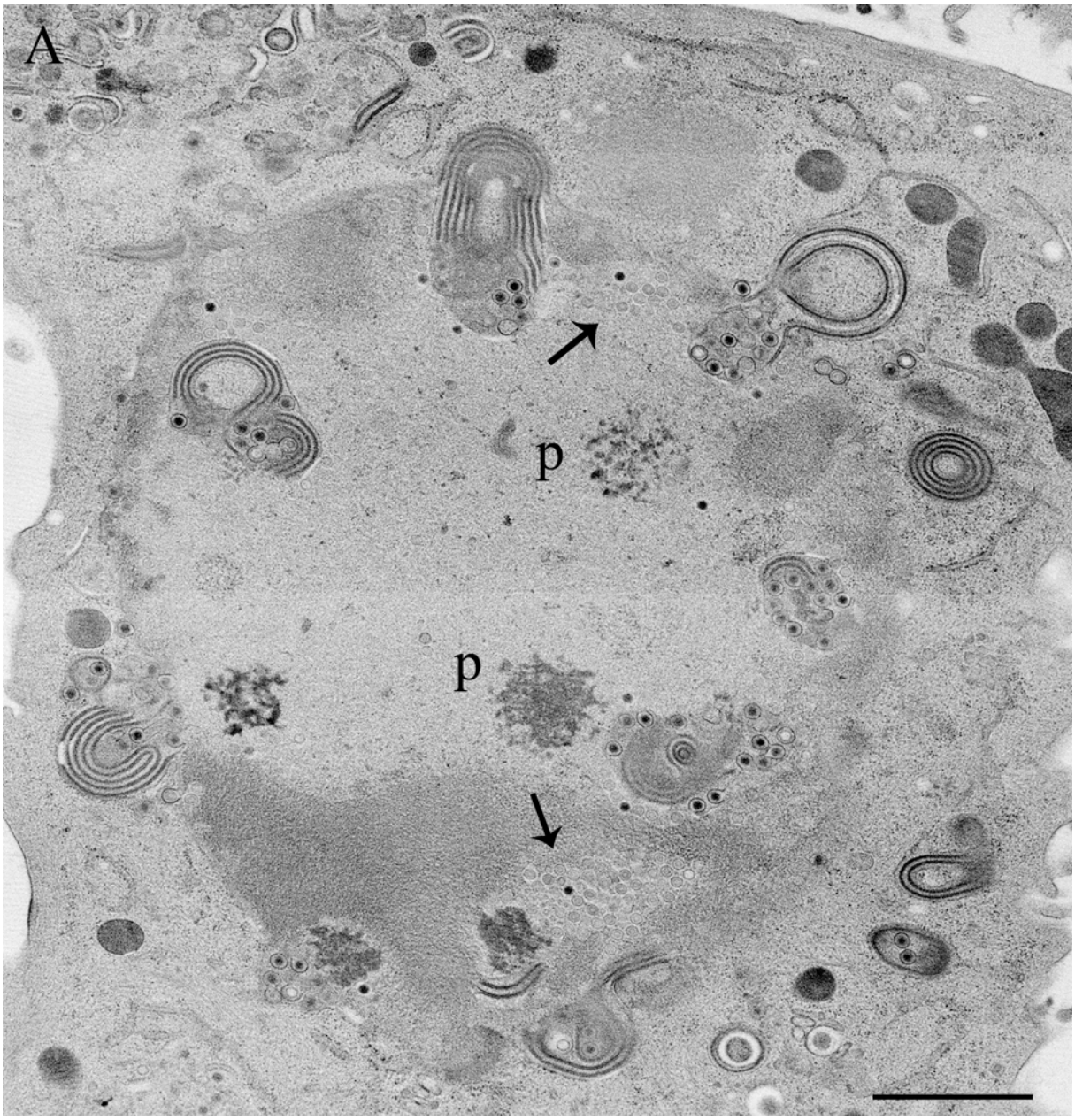

FIG. 1. Tangential section through a nucleus of a Vero cell 19 h after infection with HSV-1 ΔUS3 with multiple foldings of nuclear membranes within the nucleus or cytoplasm combined with accumulations of virus particles at various stages of envelopment. Accumulation of capsids (arrows), and dense clusters consisting probably of proteins (p). Bar 1 μm.

FIG. 2. Vero cells infected with HSV-1 ΔUS3 at 17h (A) or 19h (B) post incubation. Bars, 100 nm.

(A) Budding capsid (arrow) at the inner nuclear membrane and folding of the inner nuclear membrane within the nucleus with deposition of a dense substance of unknown nature at the luminal layer (ll) whereas the cytoplasmic layer (cl) is clearly visible.

(B) Foldings of the inner and outer (o) nuclear membrane within the cytoplasm. Virions with a dense envelope are accumulated within the perinuclear space clearly delineated by the inner nuclear membrane (i). The border (arrowheads) between nucleus and cytoplasm is poorly demarcated because the nucleus is tangentionally sectioned.

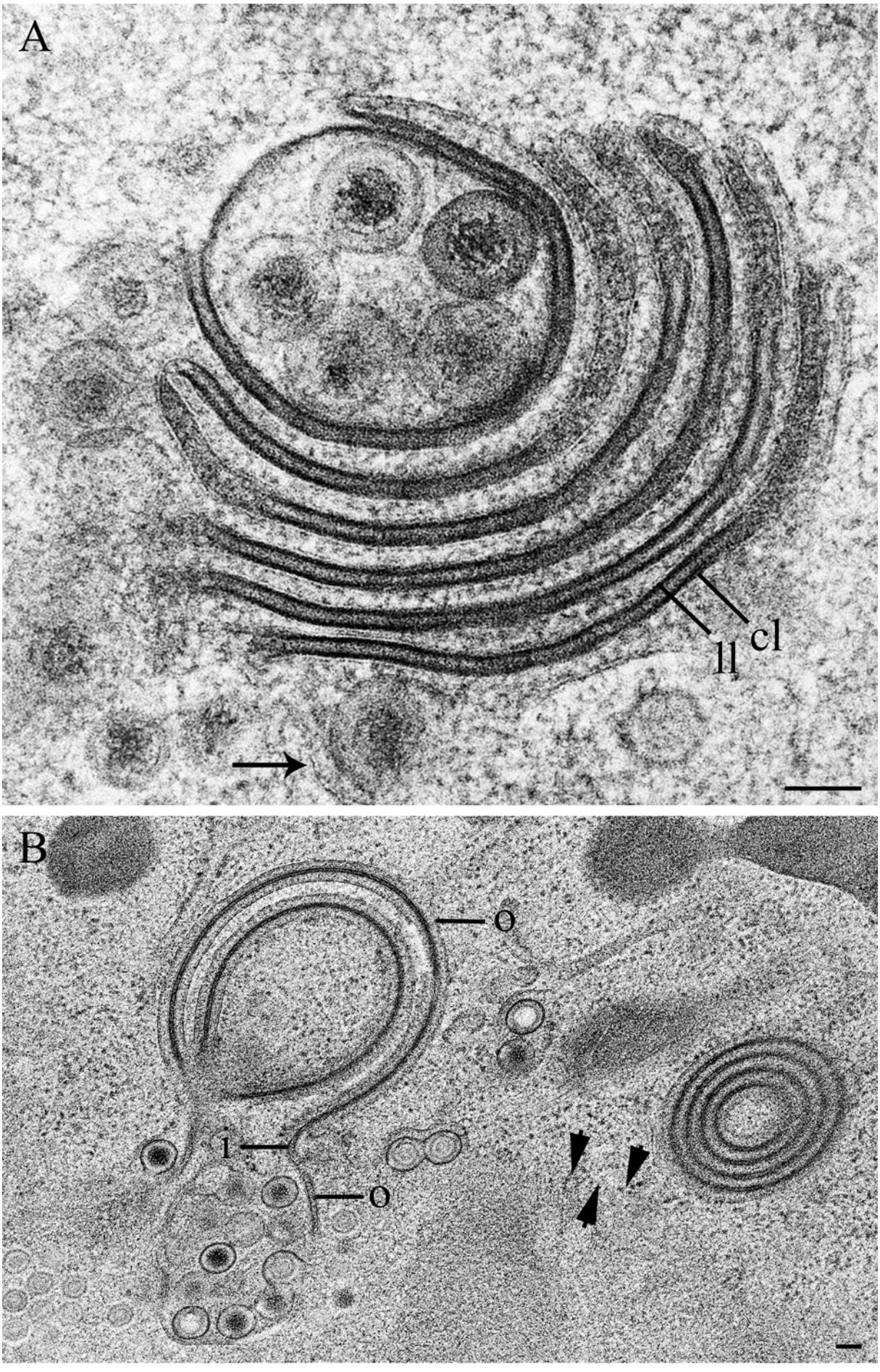
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FIG. 3. The nuclear surface of Vero cells 17 or 19 h after infection with HSV-1 ΔUS3. Bars, 100 nm

(A, B) Foldings of the inner nuclear membrane with depositions of an electron dense substance at the luminal layer and budding (arrows) of capsids at various stages at the outermost folding.

(C, D) Accumulation of virions with a dense envelope within the perinuclear space. Capsids bud at the inner nuclear membrane (i). The inner dense nuclear membrane is folded into the cytoplasm together with the outer nuclear membrane (o). The perinuclear space is delineated to the nucleus by the inner nuclear membrane. np: nuclear pore

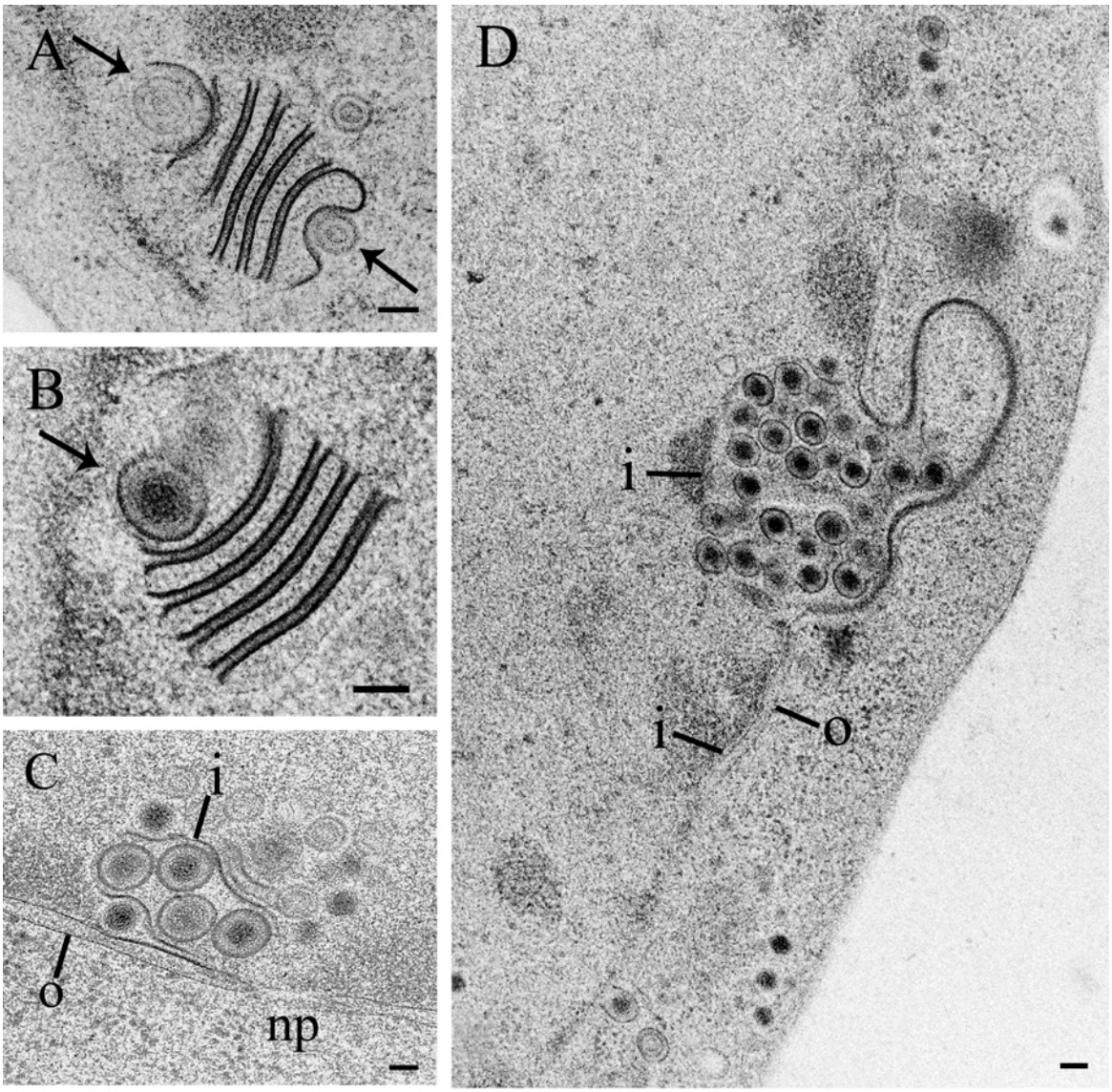
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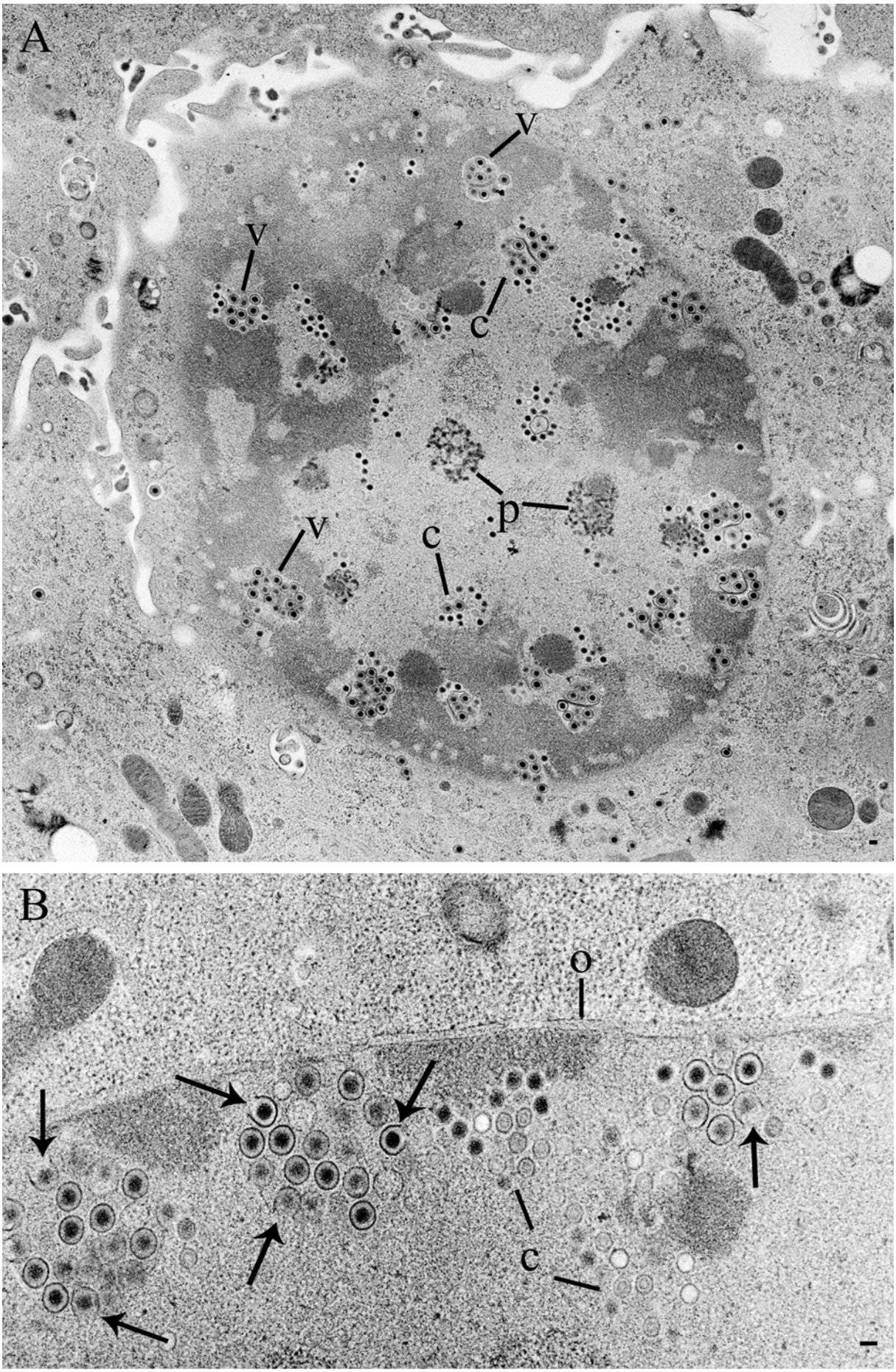
FIG. 4. Vero cells incubated with HSV-1 ΔUS3 at 17 or 19 h. Bars, 100 nm.

(A) Accumulation of virions (v) probably within the perinuclear space, of capsids (c) and probably of proteins (p) within the nuclear matrix.

(B) Accumulations of virions with a dense envelope probably within the perinuclear space as indicated by budding of capsids (arrows). c: capsids;
o: outer nuclear membrane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4.1.2. Nuclear Capsids

Capsids were irregularly scattered within the nucleus often aggregating to clusters. To get an idea about the frequency of capsid clusters we determined the number of clusters per 100 μm² by counting them on 25 randomly chosen cellular profiles. In the nuclear matrix about 40 to 50 capsids were found to accumulate to clusters (Fig. 5) often in a quite geometrical manner (Fig. 5D).

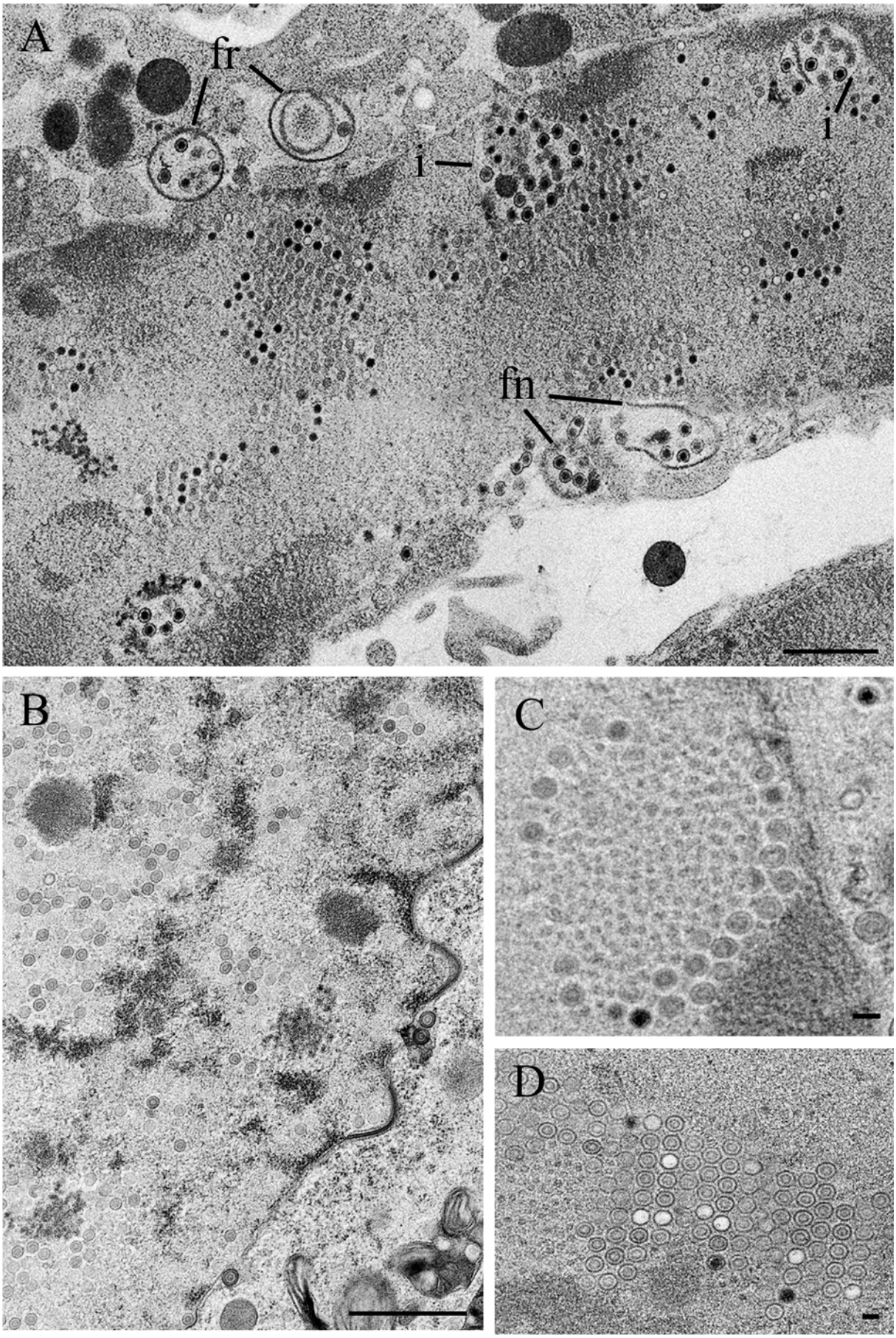
FIG. 5. Accumulations of capsids within the nucleus in Vero cells at 17 or 19 h of infection with HSV-1 ΔUS3. A, B: Bars, 1 μm; C, D: Bars, 100 nm.

(A) Large clusters of A-, B- and C-capsids within the nuclear matrix and enveloped virions within the perinuclear space clearly delineated by the inner nuclear membrane (i). Foldings of the nuclear membrane (fn) into the cytoplasm, and foldings of the RER (fr).

(B) Mainly B- capsids are scattered throughout the nuclear matrix. Note the undulated dense nuclear envelope.

(C) Accumulation of probably not fully assembled capsid structures surrounded by B-capsids.

(D) Accumulation of mainly B-capsids together with a few A- and C-capsids, and probably unassembled capsid structures.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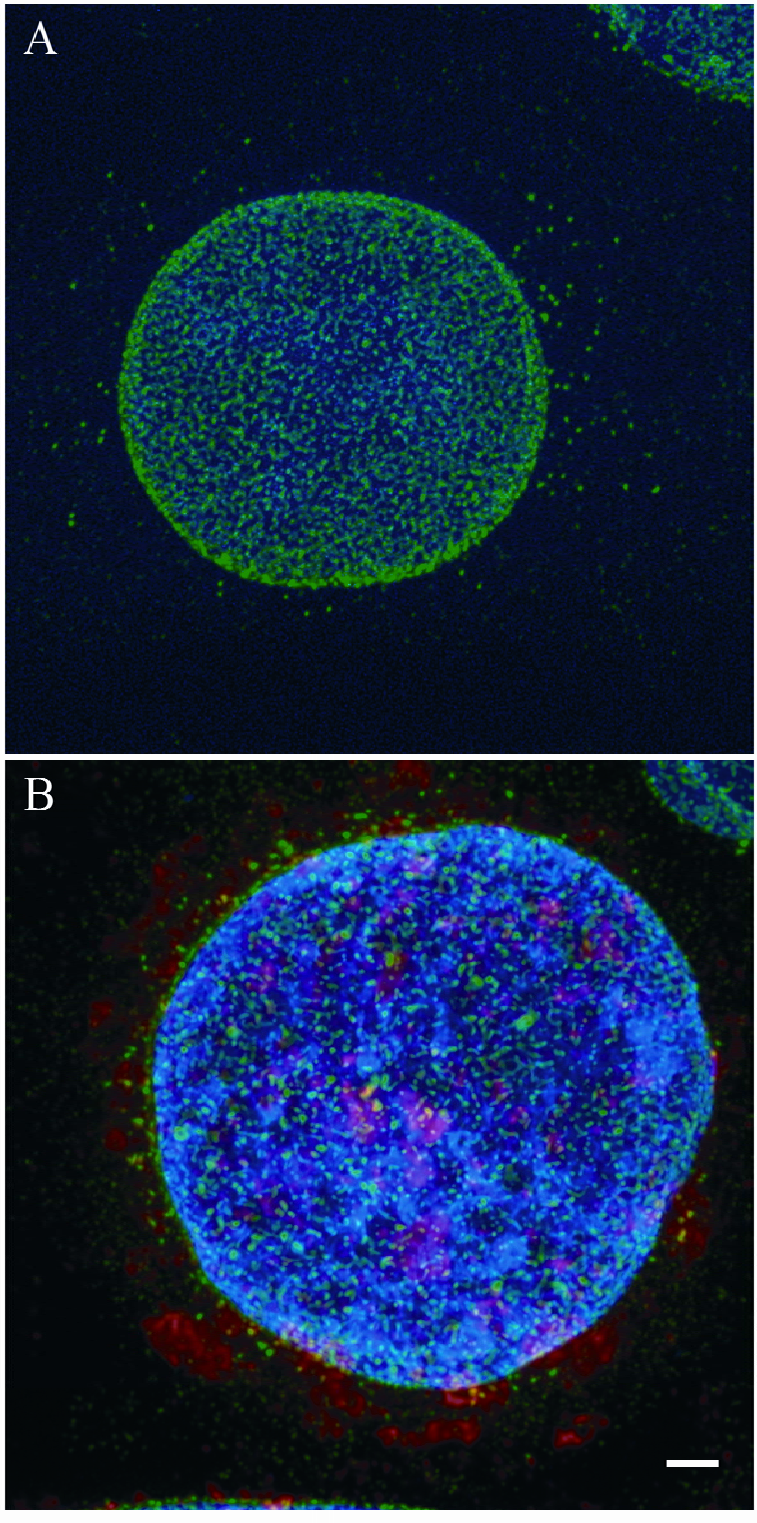
4.1.3. Nuclear Pores

Most of the nuclear pores detected by electron microscopy were intact. Some nuclear pores, however, were destroyed and nuclear material protruded into cytoplasm but did not merge with it (Fig. 7A). Capsids were not found in association with nuclear pores. Confocal microscopy using antibodies against nuclear pore proteins revealed: that its distribution is irregular in cells infected with HSV-1 ΔUS3 compared to mock infected cells (Fig. 6). Infection of cells was recognized by immunolabeling using antibodies against VP 16.

Fig. 6. Confocal imaging of nuclear pores in Vero cells using monoclonal antibodies MAb414. Bar, 3 μm

(A) Nucleus of mock infected cell after deconvolution showing regular distribution of nuclear pores (green).

(B) Deconvolved nucleus of a Vero cell incubated with HSV-1 ΔUS3 for 12 h contains irregularly distributed nuclear pores. Tegument protein VP 16 (red) is expressed in the nucleus and in the cytoplasm.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4.2. Cytoplasm

4.2.1. RER-Golgi complex

Very few virions were found in RER cisternae and in Golgi cisternae (Fig. 7B). The Golgi fields were small at 9 h of incubation (Fig. 7A) and became drastically enlarged at 17 and 21h (Fig. 7B and C) of incubation (Table I). At 17 h p.i. the volume of the Golgi complex was smaller than at 9 and 21 h whereas surface area was higher at 9 and 21 h.

In some Golgi fields we found deposition of proteins at the cytoplasmic layer probably of viral origin (Fig. 7C and D) looking similar to the substance of the viral envelope in the perinuclear space and at the inner nuclear membrane.

Table I: Volume and surface area of the Golgi complex calculated per 1000 µm^3 cytoplasm in cells infected with HSV-1 ΔUS3 at MOI 5

	9 h	**17 h**	**21 h**
Surface area	2680	7286	8866
Volume	152	126	153
Surface/ Volume	17.6	57.8	58

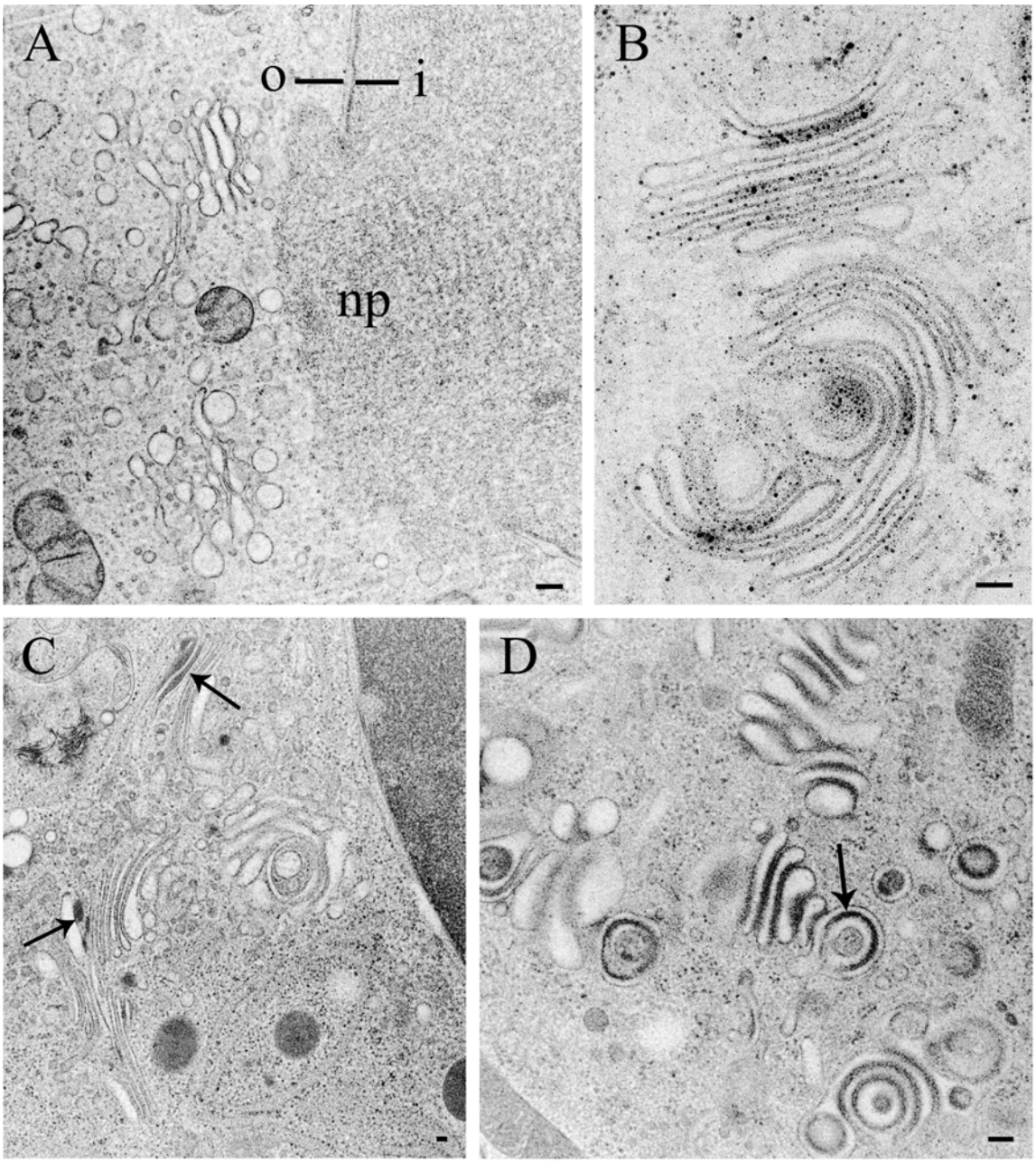

FIG. 7. Golgi complex in Vero cells incubated after inoculation with HSV-1 ΔUS3 for 9 h, 17 h and 21 h. Bars, 100 nm

(A) Small Golgi field (9 h p.i.) with moderately dilated cisternae adjacent to the nuclear matrix that protruded into the cytoplasm via a markedly dilated (4 μm) nuclear pore (np) distinctly delineated by the nuclear membranes (i,o).

(B) Large Golgi field (17 h p.i.) that is not involved in envelopment.

(C) Large Golgi field (21 h p.i.) with some deposition of proteins (arrows) most probably of viral origin.

(D) Dispersed Golgi cisternae at 17 h p.i. with deposition of proteins (arrow) probably of viral origin.

4.2.2. Distribution of Phenotypes

In an attempt to get an idea about the distribution of the various phenotypes viral particles within the cytoplasm were counted on images taken at random between 17 and 21 h post inoculation. Table II shows that the majority of phenotypes were at the inner nuclear membrane and within the perinuclear space. The number was by a factor of 2 higher at 17 h compared to 20 and 21 h. The number of naked capsids within the cytoplasm, however, was more than 6 and 5 folds higher, respectively at 20 and 21 h compared to 17 h. The number of virions within vacuoles (Fig. 8) assumed to derive by packaging (P-vacuoles) was low whereas the number of virions within vacuoles assumed to derive by wrapping was about 7 fold higher at 17 and 21 h and even 16 fold higher at 20 h compared to P-vacuoles.

FIG. 8. Virus particles in Vero cells at 19 h of incubation with HSV-1 ΔUS3. Bars, 100 nm.

(A) Naked C- and B-capsids within the cytoplasm. Some are involved in budding at membranes of possible Golgi origin. Virions within vacuoles probably derived by wrapping (w) and by packaging (p) for transportation to the plasma membrane.

(B) Large Golgi complex and a laterally dilated cisterna containing one virion with a dense substance at the envelope suggesting its nuclear origin.
p: packaging vacuole

(C) Golgi membranes with a capsid budding at its membrane (b), and a virion within a vacuole probably derived by packaging (p).

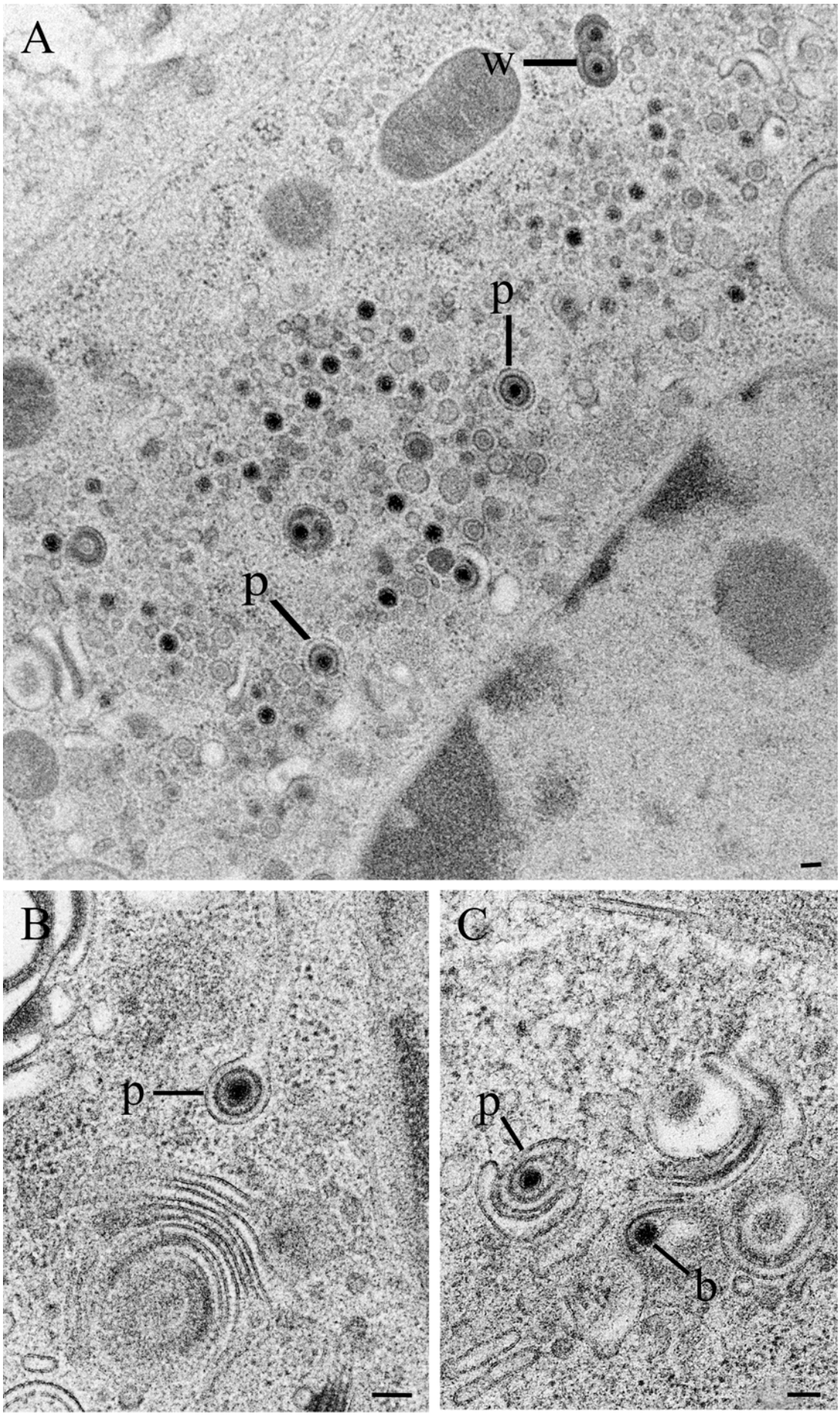
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Table II: Number of virus particles expressed per 100 µm^2 of cytoplasm in Vero cells incubated for 17, 20 and 21 h after infection with HSV-1 at MOI 5

US3				wt
Phenotypes	**17 h**	**20 h**	**21 h**	**17 h**
Virus particles at INM and PNS	112.1	60.6	55.1	4
Virions in P vacuoles	2.1	1.8	1.1	4
Nuclear envelopment	114.2	62.4	56.2	8
Naked capsids	4.6	31.2	24.1	3
Virions in W vacuoles	14.9	31.4	14.3	6
Cytoplasmic envelopment	19.5	62.5	38.4	9
Ratio cytoplamic to nuclear envelopment	1: 5.9	1: 1	1: 1.5	1: 0.9
Nuclear clusters of capsids	4.2	7.4	6.8	1

INM: inner nuclear membrane; **PNS**: perinuclear space; **P-vacuoles**: large vacuoles derived by packaging (Fig. 8); **W-vacuoles**: derived by wrapping: small vacuoles with one virion and an electron-dense substance between envelope and vacuolar membrane.

4.3. Virus Yields

PFU/ml $3x10^7$ of infectious virus was determined by plaque titration. The virus yield was $1x10^7$ to $3x10^7$ similar as in Vero cells infected with wt virus.

4.4. Effects of Brefeldin A on HSV-1 ΔUS3 Envelopment

BFA causes disassembly of the Golgi apparatus that rapidly disappears as a morphologically distinct entity (Lippincott-Schwartz, Yuan et al. 1989; Lippincott-Schwartz, Donaldson et al. 1990). Treatment of wt HSV-1 infected cells with BFA was shown to inhibit transportation of virions out of the perinuclear space (Reinhardt 2006). To test whether impaired functionality of the Golgi complex influences envelopment at the inner nuclear membrane we used BFA in concentrations of 1 µl/ml added at 5 or 8 h post inoculation. Surprisingly, after BFA treatment the budding of capsids at the inner nuclear membrane and the number of virions in the perinuclear space were lower than in untreated cells (Fig. 9) whereas the number of virions within RER cisternae was substantially increased indicating that the virions were transported into RER cisternae (Fig. 10). The foldings of the inner nuclear membrane were less and the outer nuclear membrane was not folded. There was an intense proliferation of RER membranes. The envelope of virions within RER cisternae had a similar dense substance as those in the perinuclear space (Fig. 11).

Fig. 9. Tangential section through a cell nucleus infected with HSV-1 ΔUS3 and after treatment with BFA at 8 h p.i. Small foldings of the nuclear membrane within the nucleus (fn) and proliferations of RER membrane (fr). The contour of the nucleus is poorly visible. Most virions (v) got stuck in the perinuclear space-RER compartment. Bar, 1 μm

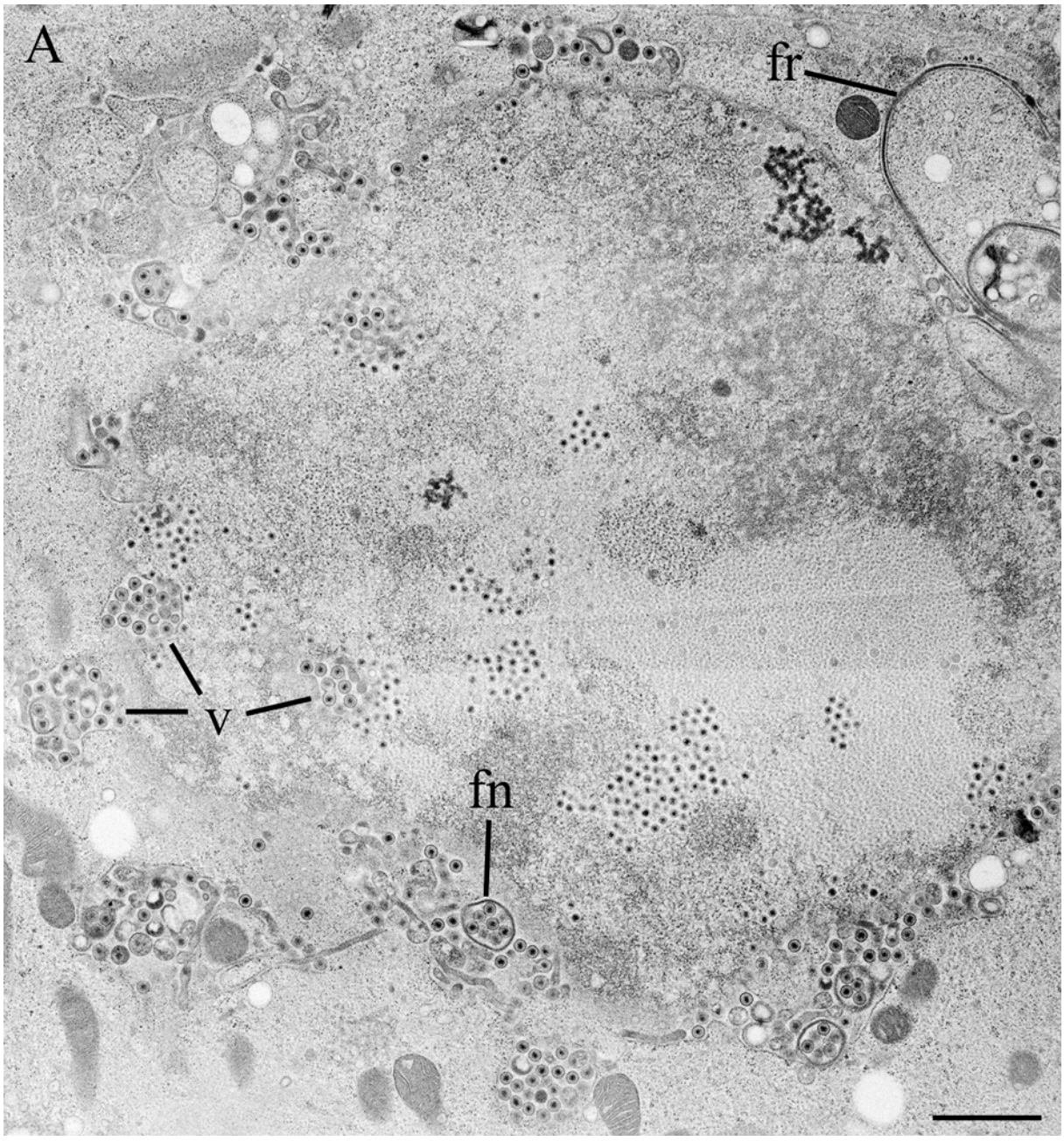
A
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Fig 10. Vero cells at 17 h of incubation with HSV-1 ΔUS3 and after addition of BFA at 5 h p.i. Bars, 1μm.

(A, B) Proliferation of RER membranes (fr) and accumulations of large numbers of virions (v) predominantly within dilated RER cisternae.

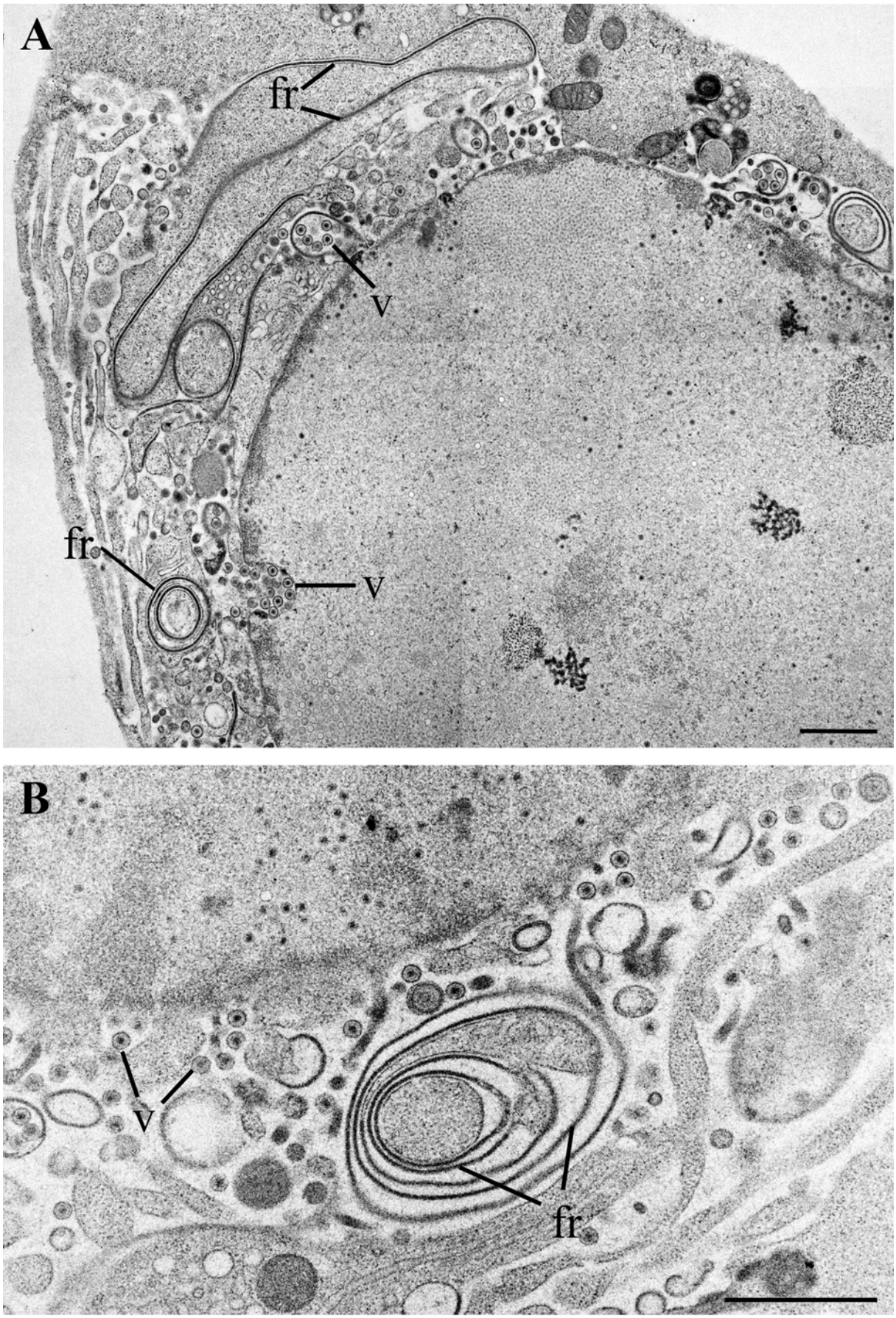
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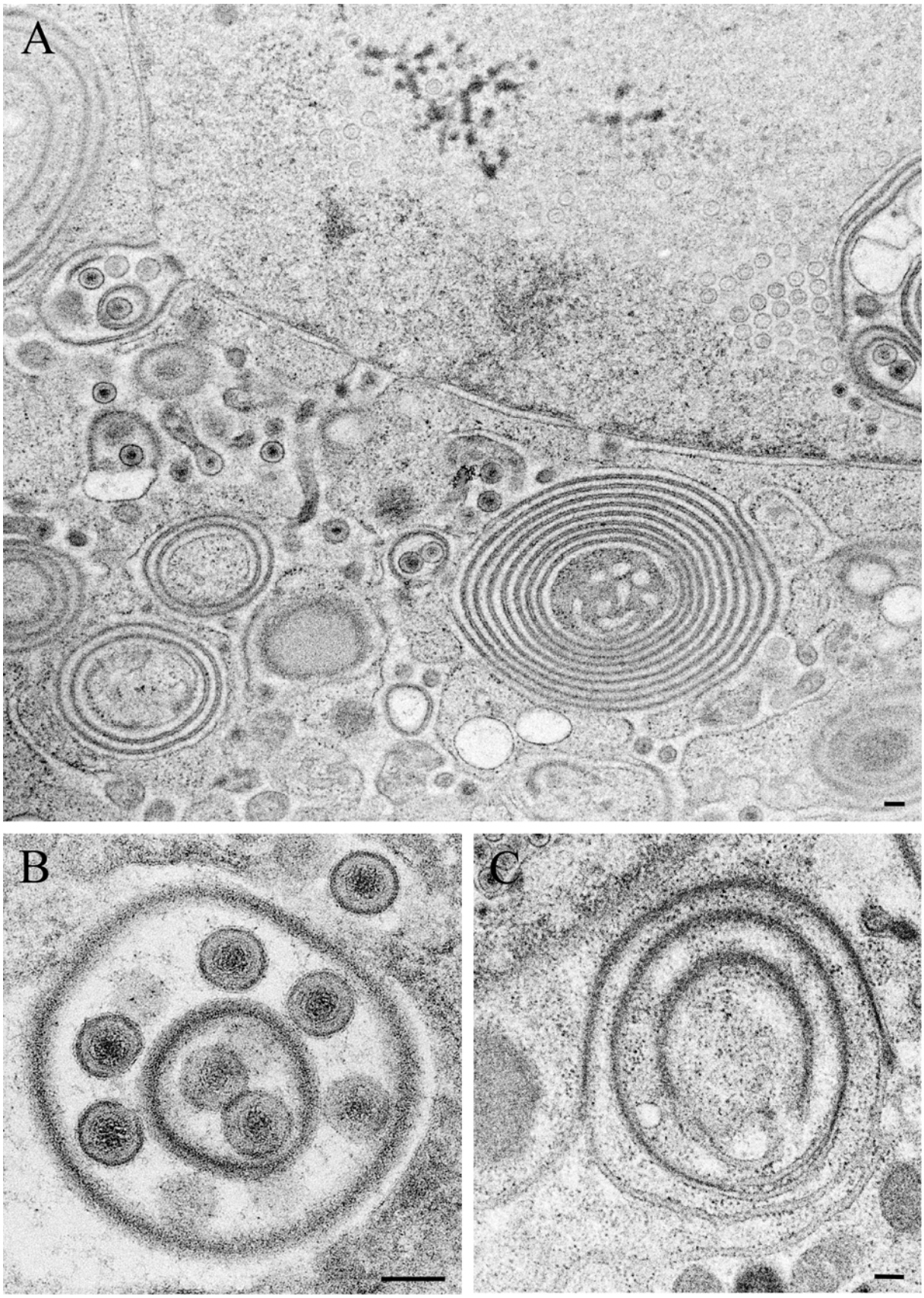
Fig. 11. Vero cells infected with HSV-1 ΔUS3 for 18 h and after treatment with BFA at 8 h p.i. Bars, 100nm.

(A) Foldings of the RER and dilation of RER cisternae containing enveloped virions.

(B) Accumulation of virions between proliferated RER membranes distinctly thickened by a dense substance.

(C) Proliferation of thickened membranes clearly showing its RER origin.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5. Discussion

High resolution microscopy of HSV-1 ΔUS3 infected cells revealed: (i) budding of numerous capsids at the inner nuclear membrane, (ii) accumulations of virions within the perinuclear space, (iii) intense folding of the inner nuclear membrane accompanied by deposition of a dense substance at the luminal layer, (iv) clusters of capsids within the nucleus, (v) dilation of nuclear pores, (vi) enlargement of the Golgi complex with deposition of a dense substance at the cytoplasmic layer, (vii) the presence of naked capsids within the cytoplasm, and (viii) the dominant presence of vacuoles containing a single virion assumed to have originated by budding at Golgi membranes. BFA treatment resulted in (ix) reduced number of budding capsids at the inner nuclear membrane and lower accumulation of virions in the perinuclear space but in (x) accumulation of virions within RER cisternae and (xi) intense prolieferation of RER membranes.

A crucial phenomenon in cells infected with HSV-1 ΔUS3 is the high number of budding capsids at the inner nuclear membrane and the accumulation of virions in the perinuclear space. Budding of capsids at the inner nuclear membrane is generally accepted as the pathway of capsids to exit the nucleus. The resulting virus particle is a fully enveloped virion containing tegument (Granzow, Klupp et al. 2004; Leuzinger, Ziegler et al. 2005). The high number of budding capsids in HSV-1 ΔUS3 infected cells is either caused by retardation of the budding process or by an increased production of capsids. The first idea seems to be more attractive because the onset of budding is much later in HSV-1 ΔUS3 compared to wt HSV-1 infected cells that occurred as early as 6 h p.i. (Leuzinger, Ziegler et al. 2005) and because capsids also accumulated within the nucleus often to large clusters. Accumulation of virions within the perinuclear space of cells infected with HSV-1 ΔUS3 was already described (Reynolds, Wills et al. 2002) whereas very recently these accumulations has been suggested

to be within vacuoles formed by the inner nuclear membrane (Poon, Benetti et al. 2006). We clearly show that the inner nuclear membrane intended deeply into the nucleus (Fig. 1) giving the impression of vacuoles (Fig. 3) depending on the section plane. The outer nuclear membrane, however, is not involved in the formation of these foldings.

Accumulation of virions within the perinuclear space indicates that transportation out of it is inhibited. In the absence of the PrV US3 protein homologue, large numbers of enveloped virions appeared to accumulate within invaginations of the nuclear membrane. This observation led to the deduction that the US3 encoded kinase is important for the efficient de-envelopment of nascent virions by fusion of the viral envelope with the outer nuclear membrane (Wagenaar, Pol et al. 1995; Klupp 2001). We found a low number of virions within Golgi cisternae indicating that a few virions were intraluminally transported out of the perinuclear space. We, therefore, exposed cells to BFA added 5 or 8 h post inoculation with HSV-1 ΔUS3. Surprisingly, treatment with BFA resulted in a reduction of budding capsids at the inner nuclear membrane and of virions within the perinuclear space whereas the number of virions in adjacent RER cisternae was substantially increased clearly indicating that virions exit the perinuclear space by intraluminal transportation as have been shown for wildtype HSV-1 (Schwartz and Roizman 1969; Whealy, Card et al. 1991; Gilbert, Ghosh et al. 1994; Radsak, Eickmann et al. 1996; Granzow, Weiland et al. 1997; Roller, Zhou et al. 2000; Leuzinger, Ziegler et al. 2005). The Golgi complex disintegrates within minutes after administration of BFA to cell cultures (Hess, Muller et al. 2000). The regained ability of virions due to the lack of US3 for intraluminal transportation after dissociation of Golgi membranes suggests that the primary action site of US3 kinase is at the level of the Golgi complex. This idea is strongly supported by the fact that the foldings at the inner nuclear membrane is drastically reduced in HSV-1 ΔUS3 infected

cells after treatment with BFA indicating that US3 is involved in the regulation of distribution and possibly of synthesis of membrane constituents required for envelopment, possibly in concert with facilitation of viral transport out of the perinuclear spyce. De novo synthesized phospholipids have been shown to be inserted into nuclear membranes of PrV infected cells (Ben-Porat and Kaplan 1971; Ben-Porat and Kaplan 1972) and HSV-1 infected cells (Sutter 2006). It is thus reasonable to assume that the dramatic foldings of the inner nuclear membrane is the result of enhanced phospholipid synthesis and/or retarded budding resulting in an imbalance of supply and demand of membrane constituent for envelopment. This seems to be also true for membranes of the Golgi complex which becomes enlarged by a factor of about 3 at 17 and 21 h respectively, compared to 9 h of infection. In the context of the de-envelopment theory this substantial enlargement would be explained by the lack of need of membranes for wrapping because all the virions got stuck in the perinuclear space. Our data clearly show that capsids got access to the cytoplasm. 1/3 to 3/4 of capsids-depending on the incubation time–have to acquire tegument and an envelope by budding at Golgi membranes. If the lack of US3 inhibits de-envelopment (Klupp 2001; Reynolds, Wills et al. 2002; Granzow, Klupp et al. 2004) these naked capsids within the cytoplasm will have to take another route from the nucleus to the cytoplasm then via fusion at the outer nuclear membrane. The only alternative on the basis of the current knowledge is via impaired nuclear pores as reported for HSV-1 (Leuzinger, Ziegler et al. 2005) and BHV-1 (Wild, Schraner et al. 2002).

Budding capsids at the inner nuclear membrane was shown to start by thickening of the membrane (Wild, Schraner et al. 2002; Leuzinger, Ziegler et al. 2005) due to deposition of an electron dense substance. This substance remains on the viral envelope within the perinuclear space. The significance of this substance is speculated to be responsible for the ability of intraluminal

transportation (Leuzinger, Ziegler et al. 2005), possibly, in combination with UL31 and UL 34. UL31 and UL34 proteins form a complex that accumulates at the nuclear membrane. It is assumed that this protein complex plays an important role in envelopment of capsids (Reynolds, Ryckman et al. 2001). US3 is suggested to be required for the distribution of UL31 and UL34 proteins throughout the nuclear rim (Reynolds, Ryckman et al. 2001). One possibility is that UL34 protein interacts directly with capsids and/or tegument components at the nuclear membranes (Reynolds, Ryckman et al. 2001). The UL34 protein is possibly responsible for recruiting other viral or cellular factors to the site of envelopment. Other proteins including gK and UL11 are implicated in the initial budding of herpesvirus capsids at the inner nuclear membrane (Baines and Roizman 1992). It is thus reasonable to assume that the dense substance at the viral envelope in the perinuclear space is a complex of UL11, UL31, UL34, gK and possibly other substances.

The question arises whether the substances incorporated into the folded inner nuclear membrane in HSV-1 ΔUS3 infected cells are identical to that of the viral envelope in the perinuclear space. Interestingly, the dense substance at the nuclear foldings seems always to be inserted at the luminal layer. Budding takes place at the cytoplasmic site that becomes thickened during the budding process. A very early event in budding is thickening of the membrane at the budding front by insertion of an electron dense substance starting at the luminal layer suggesting that the mechanism of deposition is similar and possibly the composition as well. To clarify the nature of the substances involved in budding on the one hand, and in thickening of the folded inner nuclear membrane on the other, immunolabeling studies at the electron microscopic level must be performed.

Large amounts of dense substances were found also to be deposited at the cytoplasmic layer of the Golgi complex filling the space between two adjacent cytoplasmic layers, i.e. exactly at the opposite layer as at the inner nuclear membrane. The difference in location to the substances at the inner nuclear membrane and at the viral envelope in the perinuclear space suggests different composition and function of the substance. Possibly, the bulk of the substance consists of tegument proteins that need also to be investigated by immunogoldlabeling.

After treatment with BFA there were intense proliferation of RER membranes whereas nuclear foldings were almost absent. A dense substance was deposited between two adjacent cytoplasmic layers similar as between Golgi membranes. Dissociated Golgi membranes are rearranged in the RER compartment (Lippincott-Schwartz, Yuan et al. 1989; Lippincott-Schwartz, Donaldson et al. 1990). We, therefore, assume that the proliferation of RER membranes is due to a membrane shift from the Golgi complex, possibly together with the dense substances. Immunolabeling studies will probably shed some light to the origin of these substances.

US3 is not essential for the production of infectious virions (Reynolds, Wills et al. 2002). Compared to wt infected cells, the virus yield was similar in the range of 1-3x10^7 PFU/ml. The most interesting question is thus where do all these virions that cannot escape the perinuclear space (Reynolds, Wills et al. 2002) or nuclear vacuoles (Poon, Benetti et al. 2006) acquire the requirements for infectivity? The simple answer is: in the course of budding at the inner nuclear membrane. According to table I 76 to 88% of all enveloped virus particles were within the perinuclear space. Consequently, the majority of virions contributing to the yield of infectious virus had originated by budding at the inner nuclear membrane. That means that virions acquire all essential components including

tegument proteins and glycoproteins by budding at the inner nuclear membrane as suggested (Stannard, Himmelhoch et al. 1996). Indeed, many glycoproteins have been shown to be present at nuclear membranes (Hutchinson, Roop-Beauchamp et al. 1995; Rychlowski, Rijsewijk et al. 2001). Consequently, budding of capsids leads to an enveloped virion containing glycoproteins. The deposition of the dense substance of the viral envelope impressively demonstrable in virions within the perinuclear space must, hence, been considered to cover the glycoproteins so that they cannot be detected by immunolabeling (Stannard, Himmelhoch et al. 1996). The significance of this dense substance was considered to facilitate transportation of virions out of the perinuclear space (Pol, Wagenaar et al. 1991; Leuzinger, Ziegler et al. 2005). One of these substances contributing directly or indirectly can be assumed to be US3.

In conclusion, the data obtained by high resolution microscopy confirmed the idea of retarded envelopment at the inner nuclear membrane in US3 deleted HSV-1 mutants. Furthermore, they clearly show, i) that large amounts of membranes are needed for envelope formation by budding at the inner nuclear membrane which is not inserted into the outer nuclear membrane, ii) that virions are transported from the perinuclear space into RER cisternae, iii) that a minor proportion of capsids gain access to the cytoplasm probably via dilated pores, iv) that virions get stuck in the perinuclear space after budding of capsids at the inner nuclear membrane is completed, and v) budding of capsids at Golgi membranes is retarded. It, hence, can be concluded that the bulk of virions contributing to the total infectious virus yield originates at the inner nuclear membrane, and furthermore, that US3 is involved in the maintenance of envelope formation, possibly in concert with intraluminal virus transportation.

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CURRICULUM VITAE

Personalien	Name:	Leisinger
	Vorname:	Sabine
	Geburtsdatum:	7. April 1979
	Heimatort:	Davos
	Nationaliät:	Schweiz
	Geburtsort:	Dornach

Ausbildung	2006	Doktorandin am Veterinäranatomischen Institut der Vetsuisse Fakultät Zürich
	1999-05	Studium der Veterinärmedizin an der Universität Zürich
	1995-99	Schweizerische Alpine Mittelschule Davos mit Maturaabschluss Typus E
	1992-95	Sekundarschule Davos Platz
	1986-92	Primarschule Davos Dorf

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